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<p>(21) International Application Number: PCT/US98/01556</p> <p>(22) International Filing Date: 27 January 1998 (27.01.98)</p> <p>(30) Priority Data: 08/789,734 27 January 1997 (27.01.97) US</p> <p>(71) Applicant (for all designated States except US): UNITED STATES GOVERNMENT as represented by THE SECRETARY OF THE ARMY [US/US]; Intellectual Property Law Division, OTJAG, DA, Suite 713, 901 North Stuart Street, Arlington, VA 22203-1837 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): SETTERSTROM, Jean, A. [US/US]; 700 Hampton Trace Lane, Alpharetta, GA 30201 (US). VAN HAMONT, John, R. [US/US]; 160A Gardiner Loop, West Point, NY 10996 (US). REID, Robert, H. [US/US]; 10807 McComas Court, Kensington, MD 20895 (US). JACOB, Elliot [US/US]; 11529 Dafford Lane, Silver Spring, MD 20902 (US). JEYANTHI, Ramasubbu [US/US]; 9725 Clocktower Lane #301, Columbia, MD 21046 (US). BOEDEKER, Edgar, C. [US/US]; 7505 Bybrook Lane, Chevy Chase, MD 20815 (US). MCQUEEN, Charles, E. [US/US]; 16805 Ethelwood Terrace, Olney, MD 20832 (US). TICE, Thomas, R. [US/US]; 1915 Forest River</p>	<p>Court, Birmingham, AL 35244 (US). ROBERTS, F., Donald [US/US]; 2 Bridge Path Circle, Dover, MA 02030 (US). FRIDEN, Phil [US/US]; 32 Washington Street, Bedford, MA 01730 (US).</p> <p>(74) Agent: BELLAMY, Werten, F., W.; Intellectual Property Law Division, Office of The Judge Advocate General, DA, Suite 713, 901 North Stuart Street, Arlington, VA 22203-1837 (US).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	
<p>(54) Title: THERAPEUTIC TREATMENT AND PREVENTION OF INFECTIONS WITH A BIOACTIVE MATERIAL ENCAPSULATED WITHIN A BIODEGRADABLE-BIOCOMPATIBLE POLYMERIC MATRIX</p> <p>(57) Abstract</p> <p>Novel burst-free, sustained release biocompatible and biodegradable microcapsules which can be programmed to release their active core for variable durations ranging from 1-100 days in an aqueous physiological environment. The microcapsules are comprised of a core of polypeptide or other biologically active agent encapsulated in a matrix of poly(lactide/glycolide) copolymer, which may contain a pharmaceutically acceptable adjuvant, as a blend of uncapped free carboxyl end group and end-capped forms ranging in ratios from 100/0 to 1/99.</p>		

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1                    THERAPEUTIC TREATMENT AND  
2                    PREVENTION OF INFECTIONS WITH A BIOACTIVE MATERIALS  
3                    ENCAPSULATED WITHIN A BIODEGRADABLE-BIOCOMPATIBLE  
4                    POLYMERIC MATRIX

5                    I. GOVERNMENT INTEREST

6                    The invention described herein may be manufactured, used and licensed  
7                    by or for the Government for Governmental purposes without the payment to  
8                    use of any royalties thereon.

9                    II. CROSS REFERENCE

10                  This application is a continuation-in-part of U.S. Patent Application  
11                  Serial No. 08/590,973 filed January 24, 1996 which in turn is a  
12                  continuation-in-part of U.S. Patent Application Serial No. 08/446,149 filed  
13                  May 22, 1995, which in turn is a continuation of U.S. Patent Application Serial  
14                  No. 590,308 dated March 16, 1984.

15                  Additionally, this application is a continuation-in-part of U.S. Patent  
16                  Application Serial No. 08/446,148 filed May 22, 1995, which in turn is a  
17                  continuation-in-part of U.S. Patent Application Serial No. 08/867,301 filed  
18                  April 10, 1992 now U.S. Patent No. 5,417,986 issued May 23, 1995, which in  
19                  turn is a continuation-in-part of U.S. Patent Application Serial No. 590,308  
20                  filed March 16, 1984.

III. FIELD OF THE INVENTION

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1           This invention relates to compositions comprising active core  
2           material(s) such as biologically active agent(s), drug(s) or substance(s)  
3           encapsulated within an end-capped or a blend of uncapped and end-capped  
4           biodegradable-biocompatible poly(lactide/glycolide) polymeric matrix useful  
5           for the effective prevention or treatment of bacterial, viral, fungal, or parasitic  
6           infections, and combinations thereof. In the areas of general and orthopedic  
7           surgery, and the treatment of patients with infectious or chronic disease  
8           conditions, this invention will be especially useful to physicians, dentists and  
9           veterinarians.

#### 10                   IV. BACKGROUND OF THE INVENTION

11           Wounds characterized by the presence of infection, devitalized tissue,  
12           and foreign-body contaminants have high infection rates and are difficult to  
13           treat.

14           To prevent infection, in bone and soft tissue systemic antibiotics must  
15           be administered within 4 hours after wounding when circulation is optimal.  
16           This has been discussed by J.F. Burke in the article entitled "The Effective  
17           Period of Preventive Antibiotic Action in Experimental Incisions and Dermal  
18           Lesions", Surgery, Vol. 50, Page 161 (1961). If treatment of bacterial  
19           infections is delayed, a milieu for bacterial growth develops which results in  
20           complications associated with established infections. (G. Rodeheaver et al.,  
21           "Proteolytic Enzymes as Adjuncts to Antibiotic Prophylaxis of Surgical  
22           Wounds", American Journal of Surgery, Vol. 127, Page 564 (1974)). Once  
23           infections are established it becomes difficult to systemically administer certain  
24           antibiotics for extended periods at levels that are safe and effective at the

1 wound site. Unless administered locally, drugs are distributed throughout the  
2 body, and the amount of drug hitting its target is only a small part of the total  
3 dose. This ineffective use of the drug is compounded in the trauma patient by  
4 hypovolemic shock, which results in a decreased vascular flow to tissues. (L.  
5 E. Gelin et al., "Trauma Workshop Report: Shock rheology and Oxygen  
6 Transport", Journal Trauma, Vol. 10, Page 1078 (1970)).

7 Additionally, infections caused by multiple-antibiotic resistant bacterial  
8 are on the up-swing and we are on the verge of a potential world-wide medical  
9 disaster. According to the Centers for Disease Control, 13,300 patients died  
10 in U.S. hospitals in 1992 from infections caused by antibiotic-resistant  
11 bacteria. Methicillin-resistant *S. aureus* (MRSA) is rapidly emerging as the  
12 "pathogen of the 90's":

13 a. Some major teaching hospitals in U.S. report that up to 40%  
14 of strains of *S. aureus* isolated from patients are resistant to methicillin. Many  
15 of these MRSA strains are susceptible only to a single antibiotic (vancomycin).

16 b. Should MRSA also develop resistance to vancomycin, the  
17 mortality rate among patients who develop MRSA infections could approach  
18 80%, thereby increasing the threat of this infectious killer.

19 Moreover, Vancomycin resistance is on the up-swing:

20 a. 20% of Enterococci are now resistant to vancomycin

21 b. In 1989, only one hospital in New York City reported  
22 vancomycin-resistant Enterococci. By 1991, the number of hospitals reporting  
23 vancomycin resistance rose to 38.

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1                   c. transfer of vancomycin-resistant gene (via plasmid) has been  
2 shown experimentally between Enterococcus and S. aureus.

3                   Many major pharmaceutical companies around the world have either  
4 completely eliminated or significantly reduced their research and development  
5 programs in the area of antibiotic research. According to a 1994 report by the  
6 Rockefeller University Workshop in Multiple Antibiotic Resistant Bacteria, we  
7 are on the verge of a "medical disaster that would return physicians back to the  
8 pre-penicillin days when even small infections could turn lethal due to the lack  
9 of effective drugs."

10                  Despite recent advances in antimicrobial therapy and improved surgical  
11 techniques, osteomyelitis (hard tissue or bone infection) is still a source of  
12 morbidity often necessitating lengthy hospitalization. The failure of patients  
13 with chronic osteomyelitis to respond uniformly to conventional treatment has  
14 prompted the search for more effective treatment modalities. Local antibiotic  
15 therapy with gentamicin-impregnated poly(methylmethacrylate) (PMMA) bead  
16 chains (SEPTOPAL TM, E. Merck, West Germany) has been utilized in  
17 Germany for the treatment of osteomyelitis for the past decade and has been  
18 reported to be efficacious in several clinical studies. The beads are implanted  
19 into the bone at the time of surgical intervention where they provide  
20 significantly higher concentrations of gentamicin than could otherwise be  
21 achieved via systemic administration. Serum gentamicin levels, on the other  
22 hand, remain extremely low thereby significantly reducing the potential for  
23 nephro- and ototoxicity that occurs in some patients receiving gentamicin  
24 systemically.

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1        Since SEPTOPAL TM is not currently approved by the Food and Drug  
2        Administration for use in the United States, some orthopedic surgeons in this  
3        country are fabricating their own "physician-made beads" for the treatment of  
4        chronic osteomyelitis. A major disadvantage of the beads, however, is that  
5        because the PMMA is not biodegradable it represents a foreign body and  
6        should be removed at about 2-weeks postimplantation thereby necessitating in  
7        some cases an additional surgical procedure. A biodegradable-biocompatible,  
8        antibiotic carrier, on the other hand, would eliminate the need for this  
9        additional surgical procedure and may potentially reduce both the duration as  
10       well as the cost of hospitalization.

11       The concept of local, sustained release of antibiotics into infected bone  
12       is described in recent literature wherein antibiotic-impregnated PMMA  
13       macrobeads are used to treat chronic osteomyelitis. The technique as currently  
14       used involves mixing gentamicin with methylmethacrylate bone cement and  
15       molding the mixture into beads that are 7mm in diameter. These beads are  
16       then locally implanted in the infected site at the time of surgical debridement to  
17       serve as treatment. There are, however, significant problems with this  
18       method. These include: 1) initially, large amounts of antibiotics diffuse from  
19       the cement but with time the amount of antibiotic leaving the cement gradually  
20       decreases to subtherapeutic levels; 2) the bioactivity of the antibiotic gradually  
21       decreases; 3) methylmethacrylate has been shown to decrease the ability of  
22       polymorphonuclear leukocytes to phagocytize and kill bacteria; 4) the beads do  
23       not biodegrade and usually must be surgically removed; and 5) the exothermic  
24       reaction that occurs during curing of methylmethacrylate limits the method to

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1 the incorporation of only thermostable antibiotics (primarily aminoglycosides).  
2 Nevertheless, preliminary clinical trials using these beads indicate that they are  
3 equivalent in efficacy to longer term (4-6 weeks) administration of systemic  
4 antibiotics.

5 In many instances, infectious agents have their first contact with the  
6 host at a mucosal surface; therefore, mucosal protective immune mechanisms  
7 are of primary importance in preventing these agents from colonizing or  
8 penetrating the mucosal surface. Numerous studies have demonstrated that a  
9 protective mucosal immune response can best be initiated by introduction of  
10 the antigen at the mucosal surface, and parenteral immunization is not an  
11 effective method to induce mucosal immunity. Antigen taken up by the gut-  
12 associated lymphoid tissue (GALT), primarily by the Peyer's patches in mice,  
13 stimulates T helper cell (Th) to assist in IgA B cell responses or stimulates T  
14 suppressor cells (Ts) to mediate the unresponsiveness of oral tolerance.  
15 Particulate antigen appears to shift the response towards the (Th) whereas  
16 soluble antigens favor a response by the (Ts). Although studies have  
17 demonstrated that oral immunization does induce an intestinal mucosal immune  
18 response, large doses of antigen are usually required to achieve sufficient local  
19 concentrations in the Peyer's patches. Unprotected protein antigens may be  
20 degraded or may complex with secretory IgA in the intestinal lumen.

21 In the process of vaccination, medical science uses the body's innate  
22 ability to protect itself against invading agents by immunizing the body with  
23 antigens that will not cause the disease but will stimulate the formation of  
24 antibodies that will protect against the disease. For example, dead organisms



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1 are injected to protect against bacterial diseases such as typhoid fever and  
2 whooping cough, toxins are injected to protect against viral diseases such as  
3 poliomyelitis and measles.

4 It is not always possible, however, to stimulate antibody formation  
5 merely by injecting the foreign agent. The vaccine preparation must be  
6 immunogenic, that is, it must be able to induce an immune response. Certain  
7 agents such as tetanus toxoid are innately immunogenic, and may be  
8 administered in vaccines without modification. Other important agents are not  
9 immunogenic, however, and must be converted into immunogenic molecules  
10 before they can induce an immune response.

11 The immune response is a complex series of reactions that can  
12 generally be described as follows:

- 13 1. the antigen enters the body and encounters antigen-presenting cells  
14 which process the antigen and retain fragments of the antigen on their surfaces;
- 15 2. the antigen fragment retained on the antigen presenting cells are  
16 recognized by T cells that provide help to B cells; and
- 17 3. the B cells are stimulated to proliferate and divide into antibody  
18 forming cells that secrete antibody against the antigen.

19 Most antigens only elicit antibodies with assistance from the T cells  
20 and, hence, are known as T-dependent (TD). These antigens, such as  
21 proteins, can be processed by antigen presenting cells and thus activate T cells  
22 in the process described above. Examples of such T-dependent antigens are  
23 tetanus and diphtheria toxoids.

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1        Some antigens, such as polysaccharides, cannot be properly processed  
2        by antigen presenting cells and are not recognized by T cells. These antigens  
3        do not require T cell assistance to elicit antibody formation but can activate B  
4        cells directly and, hence, are known as T-independent antigens (TI). Such T-  
5        independent antigens include *H. influenzae* type by polyribosyl-ribitol-phosphate  
6        and pneumococcal capsular polysaccharides.

7        T-dependent antigens vary from T-independent antigens in a number of  
8        ways. Most notably, the antigens vary in their need for an adjuvant, a  
9        compound that will nonspecifically enhance the immune response. The vast  
10       majority of soluble T-dependent antigens elicit only low level antibody  
11       responses unless they are administered with an adjuvant. It is for this reason  
12       that the standard DPT vaccine (diphtheria, pertussis, tetanus) is administered  
13       with the adjuvant alum. Insolubilization of TD antigens into an aggregated  
14       form can also enhance their immunogenicity, even in the absence of an  
15       adjuvant. Golub ES and WO Weigle, J. Immunol. 102:389, 1969). In  
16       contrast, T-independent antigens can stimulate antibody responses when  
17       administered in the absence of an adjuvant, but the response is generally of  
18       lower magnitude and shorter duration.

19       Four other differences between T-independent and T-dependent antigens  
20       are:

21       a) T-dependent antigens can prime an immune response so that a  
22       memory response can be elicited upon secondary challenge with the same  
23       antigen. Memory or secondary responses are stimulated very rapidly and  
24       attain significantly higher titers of antibody that are seen in primary responses.

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1 T-independent antigens are unable to prime the immune system for secondary  
2 responsiveness.

3 b) The affinity of the antibody for antigen increases with time  
4 after immunization with T-dependent but not T-independent antigens.

5 c) T-dependent antigens stimulate an immature or neonatal  
6 immune system more effectively than T-independent antigens.

7 d) T-dependent antigens usually stimulate IgM, IgG1, IgG2a, and  
8 IgE antibodies, while T-independent antigens stimulate IgM, IgG1, IgG2b, and  
9 IgG3 antibodies.

10 These characteristics of T-dependent vs. T-independent antigens provide  
11 both distinct advantages and disadvantages in their use as effective vaccines.  
12 T-dependent antigens can stimulate primary and secondary responses which are  
13 long-lived in both adult and in neonatal immune systems, but must frequently  
14 be administered with adjuvants. Thus, vaccines have been prepared using only  
15 an antigen, such as diphtheria or tetanus toxoid, but such vaccines may require  
16 the use of adjuvants, such as alum for stimulating optima responses.

17 Adjuvants are often associated with toxicity and have been shown to  
18 nonspecifically stimulate the immune system; thus inducing antibodies of  
19 specificities that may be undesirable.

20 Another disadvantage associated with T-dependent antigens is that very  
21 small proteins such as peptides, are rarely immunogenic, even when  
22 administered with adjuvants. This is especially unfortunate because many  
23 synthetic peptides are available today that have been carefully synthesized to

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1 represent the primary antigenic determinants of various pathogens, and would  
2 otherwise make very specific and highly effective vaccines.

3 In contrast, T-independent antigens, such as polysaccharides, are able  
4 to stimulate immune responses in the absence of adjuvants. Unfortunately,  
5 however, such T-independent antigens cannot stimulate high level or prolonged  
6 antibody responses. An even greater disadvantage is their inability to stimulate  
7 an immature or B cell defective immune system (Mond J.J., Immunological  
8 Reviews 64:99, 1982) Mosier DE, et al., J. Immunol. 119:1874, 1977).  
9 Thus, the immune response to both T-independent and T-dependent antigens is  
10 not satisfactory for many applications.

11 With respect to T-independent antigens, it is critical to provide  
12 protective immunity against such antigens to children, especially against  
13 polysaccharides such as *H. influenzae* and *S. pneumoniae*. With respect to T-  
14 dependent antigens, it is critical to develop vaccines based on synthetic  
15 peptides that represent the primary antigenic determinants of various  
16 pathogens.

17 One approach to enhance the immune response to T-independent  
18 antigens involves conjugating polysaccharides such *H. influenzae* PRP (Cruse  
19 J.M., Lewis R.E. Jr. ed., Conjugate vaccines in Contributions to Microbiology  
20 and Immunology, vol. 10, 1989) or oligosaccharide antigens (Anderson PW, et  
21 al., J. Immunol. 142:2464, 1989) to a single T-dependent antigen such as  
22 tetanus or diphtheria toxoid. Recruitment of T cell help in this way has been  
23 shown to provide enhanced immunity to many infants that have been  
24 immunized. Unfortunately, only low level antibody titers are elicited, and

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1 only some infants respond to initial immunizations. Thus, several  
2 immunizations are required and protective immunity is often delayed for  
3 months. Moreover, multiple visits to receive immunizations may also be  
4 difficult for families that live distant from medical facilities (especially in  
5 underdeveloped countries). Finally, babies less than 2 months of age may  
6 mount little or no antibody response even after repeated immunization.

7 One possible approach to overcoming these problems is to  
8 homogeneously disperse the antigen of interest within the polymeric matrix of  
9 appropriately sized biodegradable-biocompatible microspheres that are  
10 specifically taken up by GALT. Eldridge et al. have used a murine model to  
11 show that orally-administered 1-10 micrometer microspheres consisting of  
12 polymerized lactide and glycolide, (the same materials used in resorbable  
13 sutures), were readily taken up into Peyer's patches, and the 1-5 micrometer  
14 size were rapidly phagocytized by macrophages. Microspheres that were 5-10  
15 micrometers (microns) remained in the Peyer's patch for up to 35 days,  
16 whereas those less than 5 micrometer disseminated to the mesenteric lymph  
17 node (MLN) and spleen within migrating MAC-1+ cells. Moreover, the  
18 levels of specific serum and secretory antibody to staphylococcal enterotoxin B  
19 toxoid and inactivated influenza A virus were enhanced and remained elevated  
20 longer in animals which were immunized orally with microencapsulated  
21 antigen as compared to animals which received equal doses of non-  
22 encapsulated antigen. These data indicate that microencapsulation of an  
23 antigen given orally may enhance the mucosal immune response against enteric  
24 pathogens. AF/R1 pili mediate the species-specific binding of *E. coli* RDEC-1

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1 with mucosal glycoproteins in the small intestine of rabbits and are therefore  
2 an important virulence factor. Although AF/R1 pili are not essential for E.  
3 coli RDEC-1 to produce enteropathogenic disease, expression of AF/R1 to  
4 produce enteropathogenic disease, expression of AF/R1 promotes a more  
5 severe disease. Anti-AF/R1 antibodies have been shown to inhibit the  
6 attachment of RDEC-1 to the intestinal mucosa and prevent RDEC-1 disease in  
7 rabbits. The amino acid sequence of the AF/R1 pilin subunit has recently been  
8 determined, but specific antigenic determinants within AF/R1 have not been  
9 identified.

10 In the current study we have used these theoretical criteria to predict  
11 probable T or B cell epitopes from the amino acid sequence of AF/R1. Four  
12 different 16 amino acid peptides that include the predicted epitopes have been  
13 synthesized: AF/R1 40-55 as a B cell epitope, 79-94 as a T cell epitope, 108-  
14 123 as a T and B cell epitope, and AF/R1 40-47/79-86 as a hybrid of the first  
15 eight amino acids from the predicted B cell epitope and the T cell epitope. We  
16 have used these peptides as well as the native protein to stimulate the *in vitro*  
17 proliferation of lymphocytes taken from the Peyer's patch, MLN, and spleen  
18 of rabbits which have received intraduodenal priming with microencapsulated  
19 or non-encapsulated AF/R1. Our results demonstrate the microencapsulation  
20 of AF/R1 potentiates the cellular immune response at the level of the Peyer's  
21 patch, thus enhancing *in vitro* lymphocyte proliferation to both the native  
22 protein and its linear peptide antigens. CFA/I pili, rigid thread-like structures  
23 which are composed of repeating pilin subunits of 147 amino acid found on  
24 serogroups 015, 025, 078, and 0128 of enterotoxigenic E. coli (ETEC) (1-4,

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1 18). CFA/I promotes mannose resistant attachment to human brush borders  
2 (5); therefore, a vaccine that established immunity against this protein may  
3 prevent the attachment to host tissues and subsequent disease. In addition,  
4 because the CFA/I subunit shares N-terminal amino acid sequence homology  
5 with CS1, CFA/II (CS2) and CFA/IV (CS4) (4), a subunit vaccine which  
6 contained epitopes from this area of the molecule may protect against infection  
7 with various ETEC.

8       Until recently, experiments to identify these epitopes were time  
9 consuming and costly; however, technology is now available which allows one  
10 to simultaneously identify all the T cell and B cell epitopes in the protein of  
11 interest. Multiple Peptide synthesis (Pepscan) is a technique for the  
12 simultaneous synthesis of hundreds of peptides on polyethylene rods (6). We  
13 have used this method to synthesize all the 140 possible overlapping  
14 actapeptides of the CFA/I protein. The peptides, still on the rods, can be used  
15 directly in ELISA assays to map B cell epitopes (6, 12-14). We have also  
16 synthesized all the 138 possible overlapping decapeptides of the CFA/I protein.  
17 For analysis of T cell epitopes, these peptides can be cleaved from the rods  
18 and used in proliferation assays (15). Thus this technology allows efficient  
19 mapping and localization of both B cell and T cell epitopes to a resolution of a  
20 single amino acid (16). These studies were designed to identify antigenic  
21 epitopes of ETEC which may be employed in the construction of an effective  
22 subunit vaccine.

23       CFA/I pili consist of repeating pilin protein subunits found on several  
24 serogroups of enterotoxigenic E coli (ETEC) which promote attachment to

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1 human intestinal mucosa. We wished to identify areas within the CFA/I  
2 molecule that contain immunodominant T cell epitopes that are capable of  
3 stimulating the cell-mediated portion of the immune response in primates as  
4 well as immunodominant B cell epitopes. To do this, we (a) resolved the  
5 discrepancy in the literature on the complete amino acid sequence of CFA/I,  
6 (b) immunized three Rhesus monkeys with multiple i.m. injections of purified  
7 CFA/I subunit in Freund's adjuvant, (c) synthesized 138 overlapping  
8 decapeptides which represented the entire CFA/I protein using the Pepscan  
9 technique (Cambridge Research Biochemicals), (d) tested each of the peptides  
10 for their ability to stimulate the spleen cells from the immunized monkeys in a  
11 proliferative assay (e) synthesized 140 overlapping octapeptides which  
12 represented the entire CFA/I protein, and (f) tested serum from each monkey  
13 for its ability to recognize the octapeptides in a modified ELISA assay. A total  
14 of 39 different CFA/I decapeptides supported a significant proliferative  
15 response with the majority of the responses occurring within distinct regions of  
16 the protein (peptides beginning with residues 8-40, 70-80, and 126-137).  
17 Nineteen of the responsive peptides contained a serine residue at positions 2,  
18 3, or 4 in the peptide, and a nine contained a serine specifically at position 3.  
19 Most were predicted to be configured as an alpha helix and have a high  
20 amphipathic index. Eight B cell epitopes were identified at positions 3-11, 11-  
21 21, 22-29, 32-40, 38-45, 66-74, 93-101, and 124-136. The epitope at position  
22 11-21 was strongly recognized by all three individual monkeys, while the  
23 epitopes at 93-101, 124-136, 66-74, and 22-29 were recognized by two of the  
24 three monkeys.



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1       Recent advances in the understanding of B cell and T cell epitopes have  
2 improved the ability to select probably linear epitopes from the amino acid  
3 sequence using theoretical criteria. B cell epitopes are often composed of a  
4 string of hydrophilic amino acids with a high flexibility index and a high  
5 probability of turns within the peptide structure. Prediction of T cell epitopes  
6 are based on the Rothbard method which identifies common sequence patterns  
7 that are common to known T cell epitopes or the method of Berzofsky and  
8 others which uses a correlation between algorithms predicting amphipathic  
9 helices and T cell epitopes.

#### 10       V. SUMMARY OF THE INVENTION

11       This invention relates to active core materials such as biologically  
12 active agent(s), drug(s), or substance(s) encapsulated within a biodegradable-  
13 biocompatible polymeric matrix. In view of the enormous scope of this  
14 invention it will be presented herein as Phases I, II, and III. Phase I illustrates  
15 the encapsulation of antibiotics within a biodegradable-biocompatible  
16 polymeric matrix for the prevention and treatment of wound infections. Phase  
17 II illustrates the encapsulation of antigens <sup>including tumor antigens and</sup> (more specifically, oral-intestinal  
18 vaccine antigens) within a biodegradable-biocompatible polymeric matrix  
19 against diseases such as those caused by enteropathogenic organism. Phase III  
20 illustrates the use of a biodegradable-biocompatible polymeric matrix for burst-  
21 free programmable sustained release of biologically active agents, inclusive of  
22 peptides, over a period of up to 100 days in an aqueous physiological  
23 environment.

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1           Controlled drug delivery from a biodegradable-biocompatible matrix  
2           offers profound advantages over conventional drug/antigen dosing.  
3           Drugs/antigens can be used more effectively and efficiently, less drug/antigen  
4           is required for optimal therapeutic effect and, in the case of drugs, toxic side  
5           effects can be significantly, reduced or essentially eliminated through drug  
6           targeting. The stability of some drugs/antigens can be improved allowing for a  
7           longer shelf-life, and drugs/antigens with a short half-life can be protected  
8           within the matrix from destruction, thereby ensuring sustained release of active  
9           agent over time. The benefit of a continuous sustained release of drug/antigen  
10          is beneficial because drug levels can be maintained within a constant  
11          therapeutic range and antigen can be presented either continuously or in a  
12          pulsatile mode as required to stimulate the optimal immune response. All of  
13          this can be accomplished with a single dose of encapsulated drug/antigen.

14          This invention contemplates, but is not limited to, medically acceptable  
15          methods for the effective local delivery of biologically active agents that, of  
16          themselves, are directly (e.g. drugs, such as antibiotics) or indirectly (e.g.  
17          vaccine antigens) therapeutic or prophylactic. It also includes drugs/agents that  
18          elicit/modulate natural biological activity.

19          Wounds characterized by the presence of infection, devitalized tissue,  
20          and foreign-body contaminants have high infection rates and are difficult to  
21          treat. This invention describes antibiotic formulation encapsulated within  
22          microspheres of a biodegradable-biocompatible polymer that, when applied  
23          locally to contaminated or infected wounds, provides immediate, direct, and  
24          sustained (over a period up to 100 days), high concentrations of antibiotic in

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1 the wound site (soft tissue and bone). By encapsulating antibiotics and  
2 applying them directly, one can achieve a significant reduction in nonspecific  
3 binding of the drug to body proteins, a phenomena commonly observed  
4 following conventional systemic administration of free drugs. Thus, less drug  
5 is required, higher concentrations are maintained at the site of need, and  
6 efficacy is enhanced. This approach provides superior treatment over  
7 conventional systemic administration of antibiotics for wound infections  
8 because higher bacteriocidal concentrations can be achieved and maintained in  
9 the wound environment. Higher concentrations kill more bacteria.  
10 Applicants' invention for this application is described in Phase I.  
11 Furthermore, applicants reasoned that a protective mucosal immune response  
12 might be best initiated by introduction of an antigen at the mucosal surface,  
13 because unprotected protein antigens delivered in a free form may be degraded  
14 or may complex with secretory IgA in the intestinal lumen precluding entry  
15 and subsequent processing in local immune cells. The formulation of  
16 microspheres containing antigen small enough in size to be phagocytized  
17 locally in the gut was envisioned as being able to induce an elevated localized  
18 immune response. Applicants' invention for this application is described in  
19 Phase II. In summary, applicants propose using several methods for the local  
20 application of drugs including: 1) the direct application of the encapsulated  
21 drug to a surgical/traumatized area, 2) oral delivery that provides either local  
22 deposition of microencapsulated antigen/drugs at mucosal membranes or  
23 transport across these membranes to provide local adherence of  
24 microencapsulated drugs/antigen to mucosal membranes to provide sustained

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1 release of drug/antigen into soft tissue or a body cavity, and/or 3) sustained  
2 intercellular or extracellular drug/antigen release following subcutaneous  
3 injection.

4 In those instances where antibiotics are administered locally, applicants  
5 have found that the controlled release of the antibiotic from within a  
6 biodegradable-biocompatible polymeric matrix within 14 days to about 4  
7 weeks without significant drug trailing is especially useful. However, if  
8 desired, the release of a biologically active agent from a polymeric matrix  
9 comprised of an active agent and a blend of uncapped and end-capped  
10 biodegradable poly DL(lactide-co-glycolide), can be controlled over a period of  
11 1 to about 100 days without significant drug dumping or trailing. Such novel  
12 biocompatible-biodegradable microspheres developed with a burst-free  
13 programmable sustained release of biologically active agents, inclusive of  
14 polypeptides, are described in applicants' U.S. Patent Application Serial No.  
15 08/590,973 filed January 24, 1996.

16 When antibiotics are administered systemically in the conventional  
17 manner, or locally as contemplated by the applicants, the immune response to  
18 the antibiotic and the potential for hypersensitivity and/or anaphylactoid  
19 response (especially to beta-lactam antibiotics such as penicillins/ampicillin) is  
20 a clinical concern. In early studies the inventors observed a specific IgG  
21 response to ampicillin as it was released from the microencapsulated  
22 formulation (illustrated in the histogram, Figure 1 and 2). This response is  
23 reminiscent of antibody elicited by vaccine antigens in conventional vaccines.  
24 The response to vaccine antigens is known to be accentuated by the use of an

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1       adjuvant such as alum. Alum is a crude, less adaptable delivery vehicle than  
2       its counterpart, the biodegradable-biocompatible poly DL(lactide-co-glycolide),  
3       of this invention - the polymeric matrix. This knowledge stimulated additional  
4       studies relevant to the effects of sustain release of agents on the immune  
5       response.

6               There are, in general, two forms of localized delivery which can be  
7       achieved with PLGA microspheres-delivery which is localized to individual  
8       cells of the body (intracellular delivery); and delivery which is localized to  
9       tissues within a specific region of the body (localized extracellular delivery).

10              Applicants have prepared antibiotic and hepatitis vaccine formulations  
11       which functioned by delivering localized extracellular doses of their active  
12       agents. This was achieved by using relatively large microspheres which served  
13       as a depot for the drug or antigen. Their large size 40-100 microns in diameter  
14       precluded their being phagocytized or diffusing throughout the intercellular  
15       fluid compartments of the body. Their drug agent loads were thus released  
16       within their immediate vicinity which resulted in the generation of very high  
17       local concentrations of antibiotic or the release of sufficiently high  
18       concentrations of free antigen to induce an immune response.

19              The large-diameter antibiotic bearing microspheres were originally  
20       developed by applicants primarily for topical application on exposed debrided  
21       tissues of combat wounds. However, an inherent property exhibited by the  
22       antibiotics when topically applied to a wound site is the generation of  
      measurable levels of immune response. This concept of local delivery by

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1 topical application of microspheres to tissue to achieve localized concentrations  
2 of therapeutic agents was subsequently applied to the development of an oral  
3 vaccine for protection against traveler's diarrhea caused by *E. coli*. Vaccine  
4 antigen was encapsulated into microspheres whose diameters were  
5 predominantly in the 5-10 micron size range based on an understanding that  
6 microspheres of this size would not readily be either phagocytized or  
7 transported across the gut wall into the body. Ingestion of these microspheres  
8 thus constituted a localized delivery achieved by topical application of the  
9 spheres to the wall tissue of the gut. This topical application resulted in the  
10 localized trapping of a small percentage of these sphere into the Peyer's  
11 patches where the spheres proceeded to release their antigen in a localized  
12 fashion to immune cells located within the intestinal Patches.

13 The concept of localized sustained local delivery has been further  
14 extended to the delivery of analgesics and anesthetics to exposed dental pulp to  
15 control pain and inflammatory responses. Again, the PLGA microsphere used  
16 for this type of delivery are relatively large (40-100 um in diameter) and serve  
17 as a topical depot for localized extracellular release of the drug.

18 Consistent with their understanding of the inherent immunogenic  
19 properties exhibited by active core materials *in vivo*, applicants have moved on  
20 to other non-topical application methods of using their microsphere delivery  
21 system. Some of these center on the use of small diameter microspheres  
22 ranging from sub micron to under 5 microns in diameter. These spheres allow  
23 intracellular targeting of drug or antigen. They also allow for transmucosal  
24 delivery of drugs or antigens. The concept of localized delivery in these

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1 instances refers to the localized delivery of drug or agent within individual  
2 target cells of the body regardless of their location or distribution within the  
3 body. This approach is useful in development of antitubercular, antimalarial,  
4 antiviral, and antichlamydial formulations against intracellular parasites. It is  
5 also useful for the development of vaccines against intracellular parasites and  
6 for direct delivery of agents to presenting cells of the immune system.

7 Another nontopical application method of using PLGA microspheres  
8 resides in their usefulness as injectable depots for drugs intended for either  
9 localized or systemic delivery. Typically larger diameter microspheres are  
10 used for depots as these are less likely to diffuse away. The local or systemic  
11 nature of these delivery systems is, in part a function of the release rate of the  
12 drug from the depot and the diffusional and solubility characteristics of the  
13 drug being released. Cancer chemotherapeutics, systemic antibiotics, delivery  
14 of antibiotics to infected bone are potential application of this system.  
15 Additional this non-topical systemic depot application can be extended to the iv  
16 injection of cancer-agent laden microspheres to embolize and destroy a  
17 malignant tumor. Additionally, the PLGA microspheres can be used as a  
18 carrier to deliver substances useful for the in modification of cells or genes in  
19 bioengineering or genetic procedures.

20 Interest in the concept that antigens encapsulated within a  
21 biodegradable-biocompatible polymeric matrix could be formulated as a  
22 vaccine with superior efficacy over conventional vaccines, originated from the  
23 inventors' own observations that the drug, ampicillin, when sustain released  
24 from poly DL(lactide-co-glycolide) elicited antibody production. In these

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1 studies, the applicants were able to measure specific IgG antibodies to free  
2 ampicillin and to ampicillin released from microencapsulated ampicillin  
3 formulations in the sera of mice previously "treated" with the ampicillin  
4 formulations using ELISA. Numerous other studies also document the ability  
5 of beta-lactam antibiotic to elicit antibody. Selected, more recent studies  
6 whose findings are consistent with earlier discoveries made by applicants when  
7 conducting experiments with ampicillin include those by Klein et al. (1993)  
8 who detected specific IgG antibodies (IgG and IgG3 subclasses) to the B-  
9 lactam ring in patients receiving penicillin therapy, work by Nagakura et al.  
10 (1990) which detected specific antibodies to cephalexin, a B-lactam antibiotic  
11 in the sera of guinea pigs, and Auci et al. (1993) who detected benzyl  
12 penicilloyl specific IgM, IgG IgE, and IgA antibody forming cells in lymphoid  
13 cells of mice given benzyl penicilloyl-Keyhole Limpet Hemocyanin.  
14 Pharmaceutical compositions of antigens encapsulated with poly DL(lactide-co-  
15 glycolide) are described in Phase II. The microspheres of the invention allow  
16 for introduction of vaccine antigens to mucosal surfaces in particles that can be  
17 subsequently taken up locally by phagocytic cells. Such an approach for both  
18 drugs and antigens provides significant advantages in potency and efficacy over  
19 conventional systemically administered drugs or vaccines. A partial list of  
20 biologically active agents or drugs that will potentially derive significant  
21 medical benefits from this delivery system includes: antibacterial agents;  
22 peptides; polypeptides; antibacterial peptides; antimycobacterial agents;  
23 antimycotic agents; antiviral agents; antiparasitic agents; antifungal; antiyeast  
24 agents; hormonal peptides; cardiovascular agents; hormonal



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1 peptides; cardiovascular agents; narcotic antagonists; analgesics; anesthetics;  
2 insulins; steroids including HIV therapeutic drugs (including protease  
3 inhibitors) and AZT; estrogens; progestins; gastrointestinal therapeutic agents;  
4 non-steroidal anti-inflammatory agents; parasympathomimetic agents;  
5 psychotherapeutic agents; tranquilizers; decongestants; sedative-hypnotics; non-  
6 estrogenic and non-progestional steroids; sympathomimetic agents; vaccines;  
7 vitamins; nutrients; anti-migraine drugs; electrolyte replacements; ergot  
8 alkaloids; anti-inflammatory agents; prostaglandins; cytotoxic drugs; antigens;  
9 antibodies; enzymes; growth factors; immunomodulators; pheromones;  
10 prodrugs; psychotropic drugs; nicotine; antiblood clotting drugs; appetite  
11 suppressants/stimulants and combinations thereof; contraceptive agents include  
12 estrogens such as diethyl silbestrol; 17-beta-estradiol; estrone; ethinyl estradiol;  
13 mestranol; progestins such as norethindrone; norgestryl; ethynodiol diacetate;  
14 lynestrenol; medroxyprogesterone acetate; dimethisterone; megestrol acetate;  
15 chlormadinone acetate; norgestimate; norethisterone; ethisterone; melenlate;  
16 norgestimate; norethisterone; ethisterone; melengestrol; norethynodrel; and  
17 spermicidal compounds such as nonyphenoxypolyoxyethylene glycol;  
18 benzethonium chloride; chlorindanol; include gastrointestinal therapeutic agents  
19 such as aluminum hydroxide; calcium carbonate; magnesium carbonate;  
20 sodium carbonate and the like; non-steroidal antifertility agents;  
21 parasympathomimetic agents; psychotherapeutic agents; major tranquilizers  
22 such as chloropromazine HCL; clozapine; mesoridazine; metiapine;  
23 reserpine; thioridazine; minor tranquilizers such as chlordiazepoxide;  
24 diazepam; meprobamate; temazepam and the like; rhinological decongestants;

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